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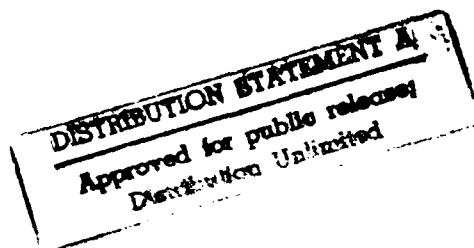
**US Army Corps
of Engineers**

Toxic and Hazardous
Materials Agency

CETHA-TS-CR-9200

**EVALUATION OF THE FEASIBILITY OF
BIODEGRADING EXPLOSIVES-CONTAMINATED
SOILS AND GROUNDWATER AT THE
NEWPORT ARMY AMMUNITION PLANT**

(NAAP)



Prepared For:

U.S. Army Toxic and Hazardous Materials Agency

Aberdeen Proving Ground, MD, 21010-5401

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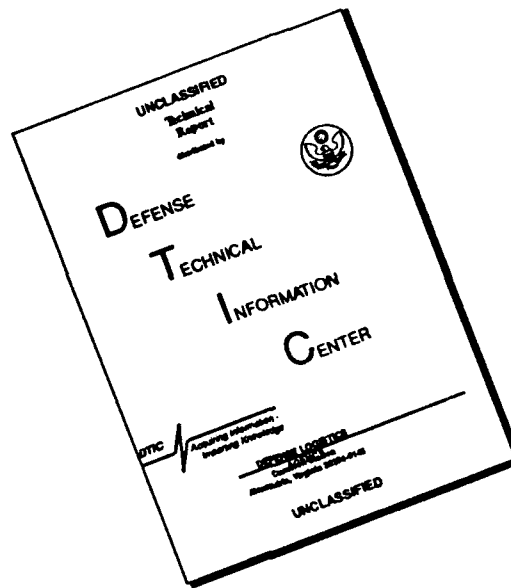
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19. of TNT. The reactors were operated in aerobic and anaerobic conditions. In no case was appreciable removal of TNT evident. A hypothesis was formed that the high concentrations of petroleum products in the NAAP soil could be interfering with TNT degradation. In one aerobic reactor, a previously isolated consortium capable of degrading petroleum products and some of the consortia isolated from JAAP were added to the system. The results clearly indicate that TNT can be biologically removed from NAAP soils, even in the presence of TPH.

To investigate the biological treatment of contaminated groundwater, a silicone tubing, fixed-film reactor was tested with two different microbial populations, Phanaerochaete chrysosporium and a bacterial based consortium. The Phanaerochaete-based reactor was operated to remove TNT from a liquid solution. In this reactor, TNT was removed from the liquid solution under nonligninolytic and ligninolytic conditions. However, this experiment was operated with a pure culture and a pure waste stream. The bacterial based reactor was operated to examine the degradation of toluene. The system removed toluene to less than 10 µg/L with very limited volatilization.

The results of this study indicate that it is feasible to biologically treat contaminated soil and groundwater at NAAP. However, more thorough investigations are necessary to obtain further, reliable, feasibility study information.

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**Evaluation of the Feasibility of Biodegrading
Explosives-Contaminated Soils and
Groundwater at the Newport Army
Ammunition Plant (NAAP)**

Prepared For:

U.S. Army Toxic and Hazardous Materials Agency
(USATHAMA)

ANL PROPOSAL P-89127

June 1991

Prepared By:

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Evaluation of the Feasibility of Biodegrading Explosives-Contaminated Soils and Groundwater at the Newport Army Ammunition Plant (NAAP)

by

C.D. Montemagno

Summary

This report summarizes the results of work conducted by Argonne National Laboratory and subcontracted to the Center for Bioengineering and Pollution Control at the University of Notre Dame. The laboratory work described in this report was conducted in support of the remedial investigation/feasibility study at Newport Army Ammunition Plant (NAAP). Laboratory studies were conducted to determine the feasibility of using biological treatment schemes to decontaminate soil and groundwater contaminated with explosives and petroleum products. The intent of this study was to determine if biological treatment has potential as a treatment alternative and to use knowledge gained in ongoing work at the Joliet Army Ammunition Plant (JAAP), which is using Soil Slurry-Sequencing Batch Reactors (SS-SBRs).

Soil samples collected from NAAP and a bacterial consortium capable of tolerating 2,4,6-trinitrotoluene (TNT) were isolated. The co-substrate succinate was required to obtain growth in the presence of TNT.

Bench scale feasibility studies were conducted to determine the ability of NAAP soil to be decontaminated in an SS-SBR. The reactors were operated in aerobic and anaerobic conditions. In no case was appreciable removal of TNT evident. A hypothesis was formed that the high concentrations of petroleum products in the NAAP soil could be interfering with TNT degradation. In one aerobic reactor, a previously isolated consortium capable of degrading petroleum products and some of the consortia isolated from JAAP were added to the system. In a period of 12 days, TNT could be removed from this reactor.

To investigate the biological treatment of contaminated groundwater, a silicone tubing, fixed-film reactor was tested with two different microbial populations, *Phanerochaete chrysosporium* and a bacterial based consortium. The *Phanerochaete*-based reactor was operated to remove TNT from a liquid solution. In this reactor, TNT was removed from the liquid solution under nonligninolytic and ligninolytic conditions. However, this experiment was operated with a pure culture and a pure waste stream. The bacterial based reactor was operated to examine the degradation of toluene. The system removed toluene to less than 10 µg/L with very limited volatilization.

The results of this study indicate that it is feasible to biologically treat contaminated soil and groundwater at NAAP. However, more thorough investigations are necessary to obtain further, reliable, feasibility study information.

1 Introduction

This document presents the results of a bench scale study to determine the feasibility of biologically treating explosives-contaminated soil and groundwater from the Trinitrotoluene Burning Ground (TNT-BG) site at Newport Army Ammunition Plant (NAAP). This report is being submitted to the U.S. Army Toxic and Hazardous Materials Agency (USATHAMA) in support of the remedial investigation/feasibility study (RI/FS) study currently being conducted by its contractor, Dames and Moore, to evaluate remedial action alternatives for the site.

The TNT-BG site encompasses approximately four acres and is located in the southwestern portion of the installation as shown in Fig. 1. The entire burning ground is enclosed by a chain link fence with a locked gate located on its north side. The burning ground consists of eastern and western burn pads and one wire mesh burn cage. The site was used for burning TNT-contaminated sludge and debris from past TNT manufacturing operations at the installation. Three burial trenches within the fenced area of the TNT-BG were reportedly used to dispose of burned explosives residues (Fig. 2).

The materials brought to the TNT-BG site for disposal by open burning included rejected TNT; TNT residue from lines and tanks at the production plant; contaminated floor sweepings; wood pallets, excelsior, and fuel oil to start the burning; and contaminated debris from the inactive plant after the plant was placed in layaway status in 1973. Several thousand gallons of liquid containing dinitrotoluene (DNT) and mononitrotoluene (MNT) are reported to have been poured directly in the trenches. A stainless steel TNT-acid separator was also reportedly buried in one trench.

The material to be burned was typically stacked on pallets on one of the burn pads. After burning, the residue was pushed by a bulldozer into a nearby trench for burial. On occasion, the burning may have been performed in the trenches themselves. In this case, the pallets would have been stacked at the bottoms of the trenches and covered with the sludge and debris, and fuel oil would have been added (if necessary) to start the burn. As in any open burning operation, complete combustion may not always have been achieved.

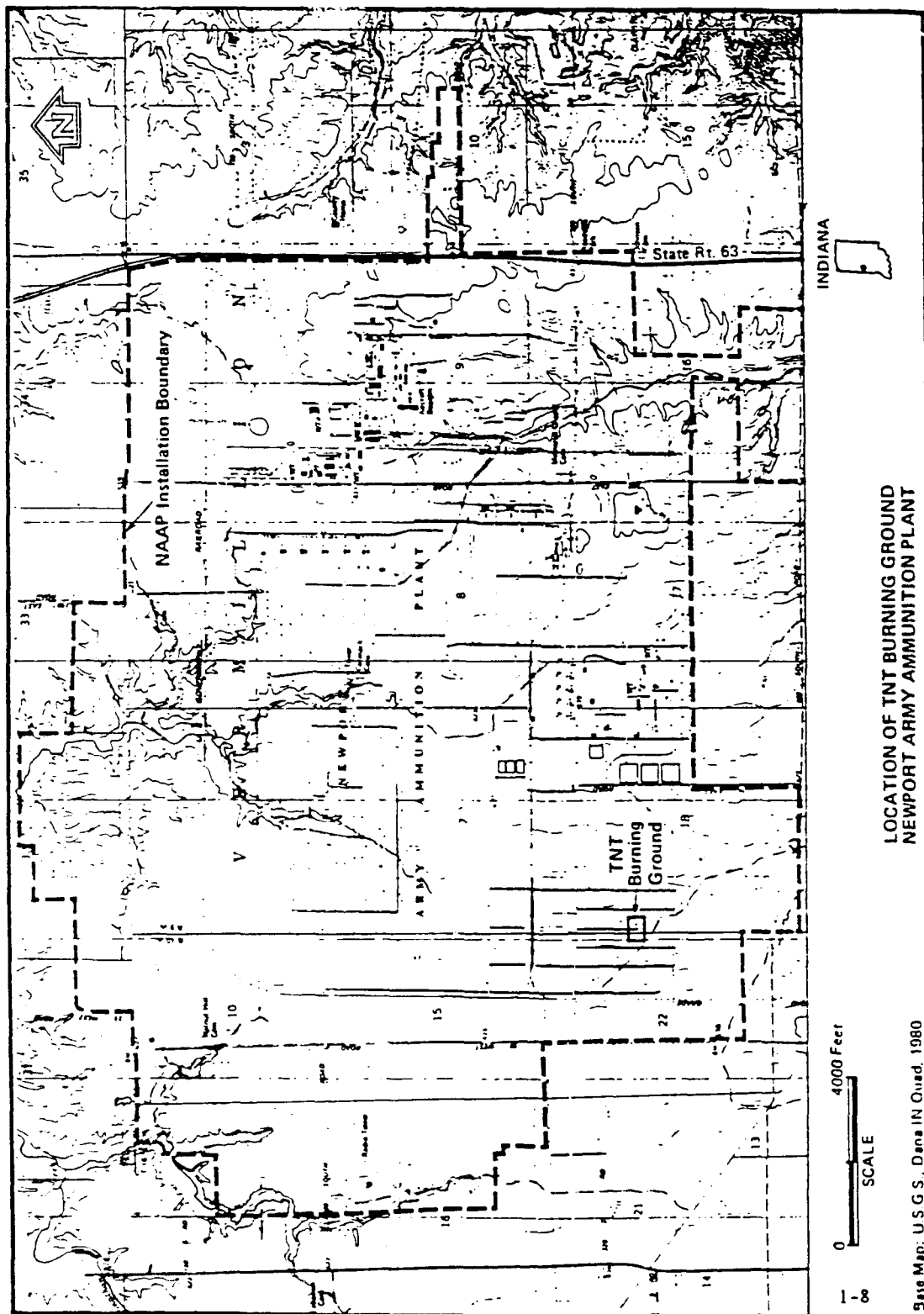


FIGURE 1 Location of TNT Burning Ground, Newport Army Ammunition Plant

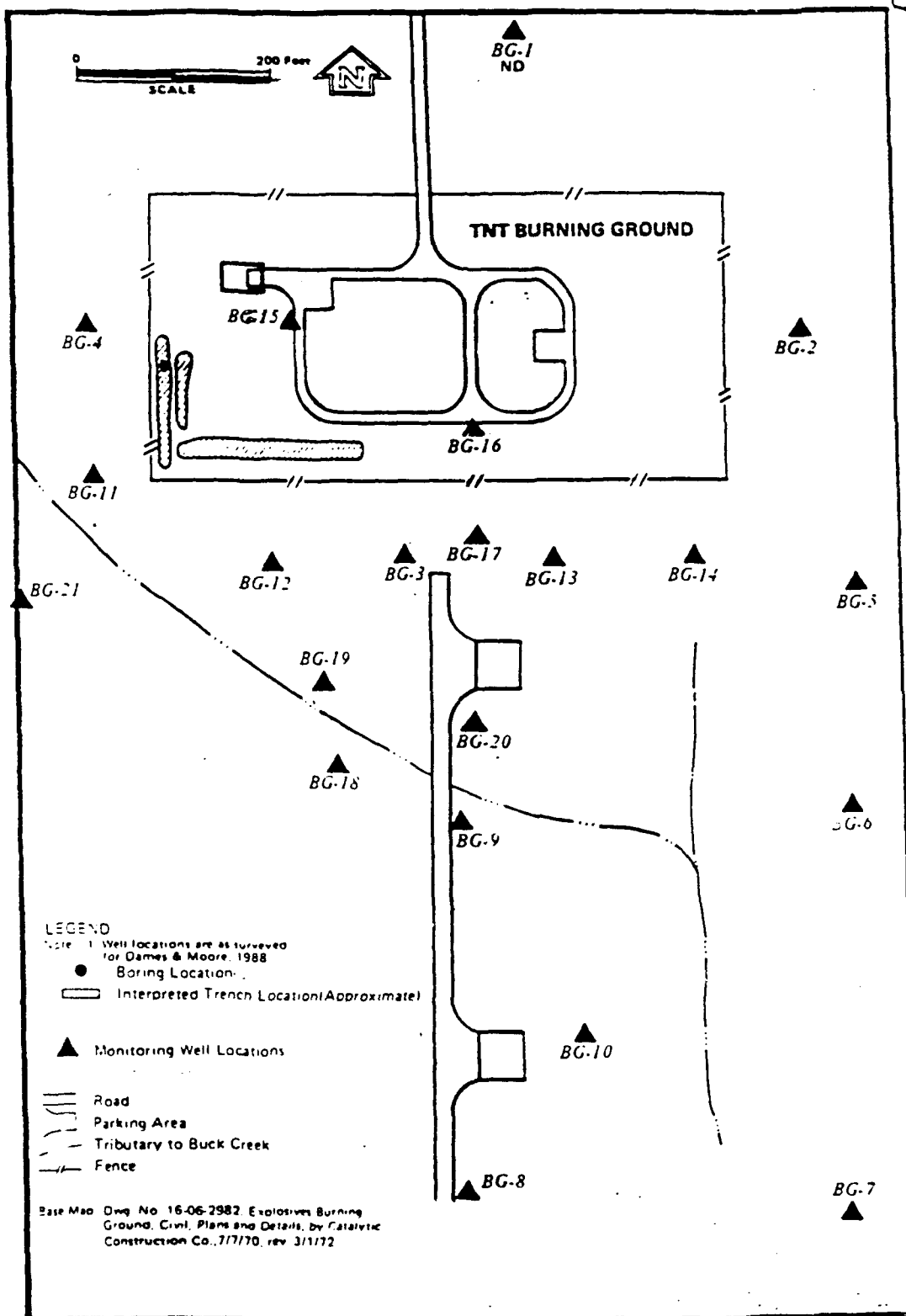


FIGURE 2 Details of TNT Burning Ground, Newport Army Ammunition Plant

The trenches were reportedly excavated by a bulldozer to typical depths of 5.6 ft, with a width equal to the width of the bulldozer blade (about 8 ft). The trenches were opened progressively as needed. A segment was seldom open for more than one month before it was backfilled. The trenches reportedly have not been used since 1973. Although open burning still takes place at the burn pads several times a year, the residue from these burns is collected and ultimately disposed of off site.

The remedial investigation currently being conducted at the site by Dames and Moore has revealed that the subsurface soil at or near the trenches is contaminated with products related to the manufacture of explosives and petroleum hydrocarbons. Contamination has been confirmed at depths of about 13-15 ft. However, the greatest concentrations of explosives have been found in soils at depths of 5-11 ft. In general, contamination has not been encountered in surface soils, but low levels of explosives were detected in two surface soil samples. Groundwater at the site is also contaminated with explosives and petroleum hydrocarbons. Site-related explosives and organics, however, have not been detected in surface water at the site. Residues from an old RDX (1,3,5-trinitro-1,3,5-triazine) manufacturing operation may have also been disposed of at the site, because the explosives RDX and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) were also detected in some of the soil and groundwater samples collected at the site. The findings of the RI and the risk assessment conducted for the site indicate that remedial action is warranted. The purpose of the feasibility study is to evaluate potential remedial action alternatives.

The purpose of this study was to evaluate the feasibility of bioremediation as a technology alternative for decontaminating the TNT-BG at NAAP. Studies were conducted to examine decontamination of explosives-contaminated soils and water by biological means. Soils obtained from NAAP were evaluated for various microbial characteristics, including growth potential and the ability to biologically degrade explosives. The study evaluated the effectiveness of microbial consortia in eliminating explosives from NAAP soil in soil slurry reactors. Consortia were (1) native organisms from NAAP and (2) organisms from an explosives contamination site at the Joliet Army Ammunition Plant (JAAP). Additional studies were conducted with *Phanerochaete chrysosporium*, a white rot fungus, and an enriched microbial consortium as a treatment alternative for TNT-contaminated groundwater. A conceptual design for treating the contaminated media at NAAP was developed.

2 Background

As a result of historic explosives manufacture and storage, large volumes of soil contaminated with TNT and related compounds (e.g., RDX) are present at numerous federal facilities. The costs associated with the remediation of these sites have been estimated to be in excess of \$1.5 billion. Studies sponsored by USATHAMA that explored both composting and land farming techniques have validated the concept of biologically decontaminating TNT-laden soils through composting. In a study directed at the development of a microbial based design concept for a periodic soil slurry reactor, explosives-contaminated soils were mixed with water (i.e., slurried) and nutrients. The design provides for the complete distribution of the target compounds, biomass, nutrients, and oxygen while it dilutes highly contaminated areas of the soil. The design approach is similar to the periodically activated sludge processes that have been developed at the University of Notre Dame.¹ These periodic processes were successfully used by Bell et al. to break down explosives.² In their study, up to 98% of the TNT present in a waste water contaminated by explosives was degraded in a 4-h cycle.

Composting is currently being considered as a viable, cost-effective, alternative treatment method for the remediation of explosives-contaminated soils. The results from bench and pilot scale studies and field demonstrations have proved that composting can biologically degrade explosives to acceptable levels.

Composting of explosives has proven to be effective, and half-lives for the breakdown of TNT have ranged from 7 to 22 d.³ Because large quantities of amendments (straw, animal feed, etc.) are used in composting, a variable fraction of the total volume composted is contaminated soil. The additives must be transported to the site and increase the final volume. The major additional component for the periodic soil slurry reactor treatment system described herein is water, which would be provided from on-site wells or local supplies. After treatment, the water is easily removed in drying beds, leaving only the treated soil.

Previous studies (Montemagno and Irvine, 1990)⁴ have shown that indigenous microbes existing at JAAP can biodegrade TNT. Soil samples collected at the JAAP site contained a bacterial consortium capable of degrading TNT. Shake flask experiments indicated that succinate or malate used as co-metabolites enhanced TNT biodegradation.

Further laboratory studies have indicated that a Soil Slurry-Sequencing Batch Reactor (SS-SBR) system has promise as a tool for bioremediating contaminated soil. Aerobic reactors reduced TNT concentrations from about 1,300 mg/L to less than 10 mg/L in 15 days. Anoxic reactors achieved the same kind of reduction but at an apparently slower rate. Bioaugmentation, the addition of microorganisms isolated from JAAP soil and having the ability to degrade TNT, did not improve the degradation rate observed in the reactors.

The advantage of the SS-SBR in treating contaminated soil is its inherent flexibility. Co-substrate, nutrients, oxygen, and mixing can be altered to achieve the desired treatment. The reactor can naturally select populations with increased degradation rates and the ability to degrade metabolic intermediates. Only naturally occurring microorganisms will be used in this study.

The current method for remediating explosives-contaminated soils is incineration.⁵ This is a costly, energy-intensive process that destroys much of the organic portion of the soil, leaving ash as the primary residue. Biological remediation would return the soil undamaged to its original location.

3 Biodegradation Studies

The NAAP soils were used for both the basic microbiological studies and the bench scale reactor studies. The microbiological studies were directed at measuring (1) the growth potential of bacteria present in the soil samples and (2) the conversion of TNT by the enriched consortium in both liquid culture and soil slurries. The bench scale reactor studies were directed at the assessment of the biological disappearance of TNT in (1) a 4-L glass kettle slurry reactor, (2) a series of 1-L reactors, and (3) a fixed-film reactor containing the white rot fungus, *Phanerochaete chrysosporium*. Additional bench scale reactor studies were conducted on the fixed-film reactor with a bacterial based consortium and toluene in the feed. Each of these studies is described in detail below.

3.1 Microbial and Sample Characterization

NAAP personnel obtained soil samples from the north portion of the western trench at the TNT-BG site (sample BG-8, Fig. 2). The samples were collected by hand and were placed in containers for overnight shipment to a commercial laboratory. Results of the laboratory analysis are presented in Table 1. These results confirm the presence of TNT and various other explosives or explosive derivative compounds.

Groundwater samples were collected from well BG-5 (Fig. 2). Samples were collected, placed in containers, and shipped overnight to a commercial laboratory. Results of the laboratory analysis are presented in Table 2. These analyses confirmed the presence of microgram quantities of TNT and various other explosives or explosive derivative compounds in the groundwater.

Two 5-gal containers of soil and one 5-gal container of groundwater simultaneously obtained from the previously described locations were transported to the University of Notre Dame. The NAAP soil had a very strong aroma resembling fuel oil and aromatic hydrocarbons. The loss on drying (i.e., water plus organics that volatilized at 103°C) of the soil was 40%. An aliquot of the moist soil was extracted for total petroleum hydrocarbons (TPH), which ranged from 2% to 3.7% in the soil samples. The TNT

TABLE 1 Analytical Results for Soil Samples Collected at NAAP

Site: PLUG BG-S						
SAMPLE DEPTH (ft)	SAMPLE DATE	TEST METHOD	COMPOUND	BOOL	CONCENTRATION	UNITS
-----	-----	-----	-----	----	-----	-----
3.0	21-aug-1990	00	BOD		3.49e+02	UGG
3.0	21-aug-1990	00	TOC		2.22e+04	UGG
3.0	21-aug-1990	99	NH3		7.61e-01	MGK
3.0	21-aug-1990	99	NO2		1.46e+01	MGKG
3.0	21-aug-1990	KF10	NIT	LT	1.50e+01	UGG
3.0	21-aug-1990	LW12	135TNB		3.11e+02	UGG
3.0	21-aug-1990	LW12	13DNB	LT	4.96e+01	UGG
3.0	21-aug-1990	LW12	246TNT		7.46e+04	UGG
3.0	21-aug-1990	LW12	24DNT		1.95e+03	UGG
3.0	21-aug-1990	LW12	26DNT	LT	5.24e+01	UGG
3.0	21-aug-1990	LW12	HMX	LT	6.66e+01	UGG
3.0	21-aug-1990	LW12	NB	LT	2.41e+02	UGG
3.0	21-aug-1990	LW12	NG	LT	4.00e+02	UGG
3.0	21-aug-1990	LW12	PETN	LT	4.00e+02	UGG
3.0	21-aug-1990	LW12	RDX	LT	5.87e+01	UGG
3.0	21-aug-1990	LW12	TETRYL	LT	7.31e+01	UGG

TABLE 2 Analytical Results for Water Samples Collected at NAAAP

Site: WELL BG-3

SAMPLE DEPTH (ft)	SAMPLE DATE	TEST METHOD	COMPOUND	BOOL	CONCENTRATION	UNITS
-----	-----	-----	-----	----	-----	-----
7.0	21-aug-1990	00	TOC		1.82e+04	UGL
7.0	21-aug-1990	99	NH3		2.16e+02	UGL
7.0	21-aug-1990	99	NO2	LT	1.00e+01	UGL
7.0	21-aug-1990	TF22	NIT		1.54e+01	UGL
7.0	21-aug-1990	TF27	PO4		1.98e+02	UGL
7.0	21-aug-1990	UW14	135TNB		1.65e+03	UGL
7.0	21-aug-1990	UW14	13DNB	LT	5.19e-01	UGL
7.0	21-aug-1990	UW14	246TNT	LT	5.88e-01	UGL
7.0	21-aug-1990	UW14	24DNT		5.03e+02	UGL
7.0	21-aug-1990	UW14	26DNT		3.39e+01	UGL
7.0	21-aug-1990	UW14	HMX	LT	1.65e+00	UGL
7.0	21-aug-1990	UW14	NB		6.29e+01	UGL
7.0	21-aug-1990	UW14	RDX	LT	2.11e+00	UGL
7.0	21-aug-1990	UW14	TETRYL	LT	5.56e-01	UGL

content (analyzed by following a USATHAMA Method, Appendix A) of the soils ranged from 0.5% to 10% (i.e., from 5,000 to 100,000 mg/kg). The detection limit for TNT in the soil was 1.92 mg/kg. The NAAP groundwater samples were clear and odorless, and no TNT was detected in them. The detection limit for TNT in the water samples was 0.5 mg/L.

Microbiological characterization studies for NAAP soils were directed at the development of consortia of microorganisms with the potential to degrade TNT; 2,4-DNT; and 2,6-DNT. NAAP groundwater and soil served as the inoculum for aerobic and anaerobic enrichments, with and without a co-substrate and/or an electron acceptor in the presence of TNT; 2,4-DNT; or 2,6-DNT.

Five grams of soil or 5 mL of groundwater was measured into a sterile 250-mL Erlenmeyer flask or a sterile 250-mL serum bottle. Then 100 mL of sterile, modified PUM mineral salts medium [16.95 g K_2HPO_4 , 7.26 g KH_2PO_4 , 3.96 g $(NH_4)_2SO_4$, and 0.098 g $MgSO_4$ per liter of solution] was added to each flask or bottle along with 100 mg/L of the target compound (TNT; 2,4-DNT; or 2,6-DNT). Serum bottles were purged with 95% CO_2 /5% N_2 and sealed. Selected flasks and serum bottles received 0.1% of the co-substrate succinate only, while other flasks and serum bottles received both succinate and 1% KN_3 . Table 3 details the various combinations. The aerobic samples were placed on a shaker at 150 rpm. The anaerobic cultures were shaken 100 rpm. All incubations were at ambient temperature. Transfers were done at one-week intervals by taking a 5-mL aliquot from the culture and placing it into an identical flask or serum bottle. All transfers employed sterile technique with pipets or syringes. After the fourth transfer, the enrichment cultures were plated on trypticase soy agar to determine the cultural diversity. The number of different colony types in each enrichment culture was determined. These data are shown in Table 3.

As can be seen from Table 3, growth occurred only when succinate was present in the medium in both the aerobic and anaerobic systems, whether or not nitrate was added as an exogenous electron acceptor. This observation was independent of the source of the inoculum (soil or water). Certain enrichment cultures containing TNT turned orange, indicating possible metabolism of TNT, like that seen for consortia from JAAP. In fact, both consortia produced the intermediates 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. Several enrichments produced a yellow color with 2,4-DNT or

TABLE 3 Enrichment Observations for NAAP Soils

Aerobic	3 transfer	4 transfer	colony types
100 mg/kg TNT (S)	no growth/clear	no growth/clear	0
100 mg/kg 2,4 DNT (S)	no growth/clear	no growth/clear	0
100 mg/kg 2,6 DNT (S)	no growth/clear	no growth/clear	0
.1%S + 100 mg/kg TNT (S)	growth/orange	growth/orange	2 or >
.1%S + 100 mg/kg 2,4 DNT (S)	growth/yellow	growth/white	2 or >
.1%S + 100 mg/kg 2,6 DNT (S)	growth/white	growth/white	2 or >
100 mg/L TNT (W)	no growth/clear	no growth/clear	0
100 mg/L 2,4 DNT (W)	no growth/clear	no growth/clear	0
100 mg/L 2,6 DNT (W)	no growth/clear	no growth/clear	0
.1%S + 100 mg/L TNT (W)	growth/orange	growth/orange	1
.1%S + 100 mg/L 2,4 DNT (W)	growth/white	growth/white	1
.1%S + 100 mg/L 2,6 DNT (W)	growth/white	growth/white	1
Anaerobic	3 transfer	4 transfer	colony types
100 mg/kg TNT (S)	no growth/clear	no growth/clear	0
100 mg/kg 2,4 DNT (S)	no growth/clear	no growth/clear	0
100 mg/kg 2,6 DNT (S)	no growth/clear	no growth/clear	0
.1%S + 100 mg/kg TNT (S)	growth/yellow	growth/yellow	1 or >
.1%S + 100 mg/kg 2,4 DNT (S)	growth/lt. yellow	growth/lt. yellow	1 or >
.1%S + 100 mg/kg 2,6 DNT (S)	growth/dk. yellow	growth/yellow	1 or >
.1%KNO ₃ + .1%S + 100 mg/kg TNT (S)	growth/yellow	growth/lt. orange	2 or >
.1%KNO ₃ + .1%S + 100 mg/kg 2,4 DNT (S)	growth/white	growth/white	2 or >
.1%KNO ₃ + .1%S + 100 mg/kg 2,6 DNT (S)	growth/lt. brown	growth/white	2 or >
.1%KNO ₃ + 100 mg/kg TNT (S)	no growth/clear	no growth/clear	0
.1%KNO ₃ + 100 mg/kg 2,4 DNT (S)	no growth/clear	no growth/clear	0
.1%KNO ₃ + 100 mg/kg 2,6 DNT (S)	no growth/clear	no growth/clear	0
100 mg/L TNT (W)	no growth/clear	no growth/clear	0
100 mg/L 2,4 DNT (W)	no growth/clear	no growth/clear	0
100 mg/L 2,6 DNT (W)	no growth/clear	no growth/clear	0
.1%S + 100 mg/L TNT (W)	growth/yellow	growth/yellow	1
.1%S + 100 mg/L 2,4 DNT (W)	growth/white	growth/white	1
.1%S + 100 mg/L 2,6 DNT (W)	growth/white	growth/white	1
.1%KNO ₃ + .1%S + 100 mg/L TNT (W)	growth/lt. brown	growth/white	1 or >
.1%KNO ₃ + .1%S + 100 mg/L 2,4 DNT (W)	growth/white	growth/white	2 or >
.1%KNO ₃ + .1%S + 100 mg/L 2,6 DNT (W)	growth/white	growth/white	2 or >
.1%KNO ₃ + 100 mg/L TNT (W)	no growth/clear	no growth/clear	0
.1%KNO ₃ + 100 mg/L 2,4 DNT (W)	no growth/clear	no growth/clear	0
.1%KNO ₃ + 100 mg/L 2,6 DNT (W)	no growth/clear	no growth/clear	0

S = Soil

W = Water

.1%S = 0.1% succinate

2,6-DNT, suggesting that some metabolism of these compounds had occurred and that para-nitrophenol may have been formed. A number of enrichments showed no growth, indicating that no organisms present in the inoculum could grow on the carbon sources provided without the addition of the co-substrate.

3.2 Bacterial Degradation of Explosives in Soils from NAAP

Montemagno and Irvine⁴ demonstrated that small amounts of radiolabeled TNT were identifiable in the microbial consortium as trichloroacetic acid- (TCA-) precipitable consortium biomass. To investigate this possibility further, four pure cultures of microorganisms, one NAAP consortium, and one JAAP consortium were grown in Stanier's medium⁶ containing uniformly radiolabeled TNT and either succinate or maleic acid as a co-substrate. As Figs. 3 and 4, show less than 15% of the original radiolabeled TNT was present as TCA-precipitable material (biomass) in all cases. On the basis of previous work (Montemagno and Irvine⁴), this indicated that small amounts of TNT were incorporated into cellular material.

It is important to note that the consortium obtained from JAAP is superior to the microbial consortia isolated from NAAP. Furthermore, the presence of high concentrations of TPH may have interfered with the ability of the microorganisms to degrade TNT.

3.3 Bench Scale Treatability Studies

Laboratory studies were conducted to determine the feasibility of decontaminating explosives-contaminated soil by using an SS-SBR. These studies were intended to be carried out in conjunction with extensive feasibility studies conducted for JAAP.

The SS-SBR is a suspended growth system that is designed to treat soil in a 15%-20% soil slurry. This reactor operates in a periodic fashion to cyclically replace 5%-20% of the fluid volume and provide for effective treatment. (A drawing of this reactor is in Appendix A.)

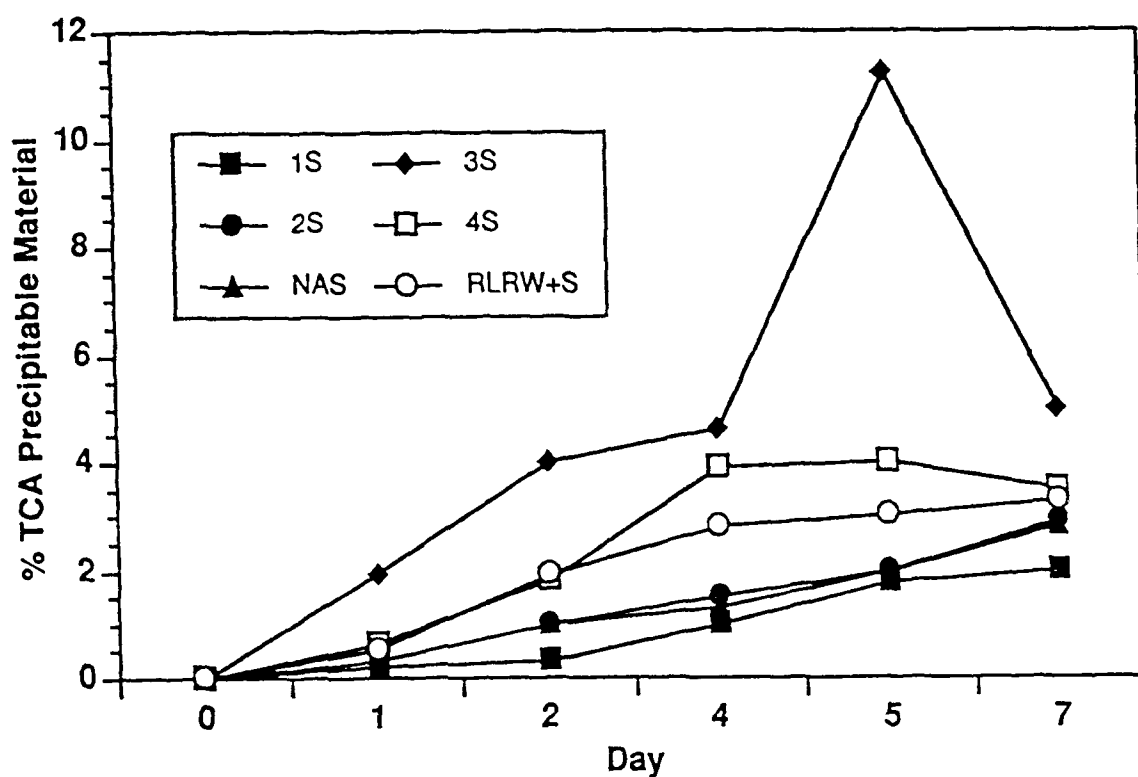


FIGURE 3 Radiolabeled TNT in TCA-Precipitable Biomass for Consortium Growing with Succinate Co-Substrate

1S - Isolate #1 with 0.5% succinate grown at 30°C.

2S - Isolate #2 with 0.5% succinate grown at 30°C.

3S - Isolate #3 with 0.5% succinate grown at 30°C.

4S - Isolate #4 with 0.5% succinate grown at 30°C.

NAS - Newport consortium with 0.5% succinate grown at 30°C.

RLRW + S - RLRW consortium from Joliet with 0.5% succinate grown at 30°C.

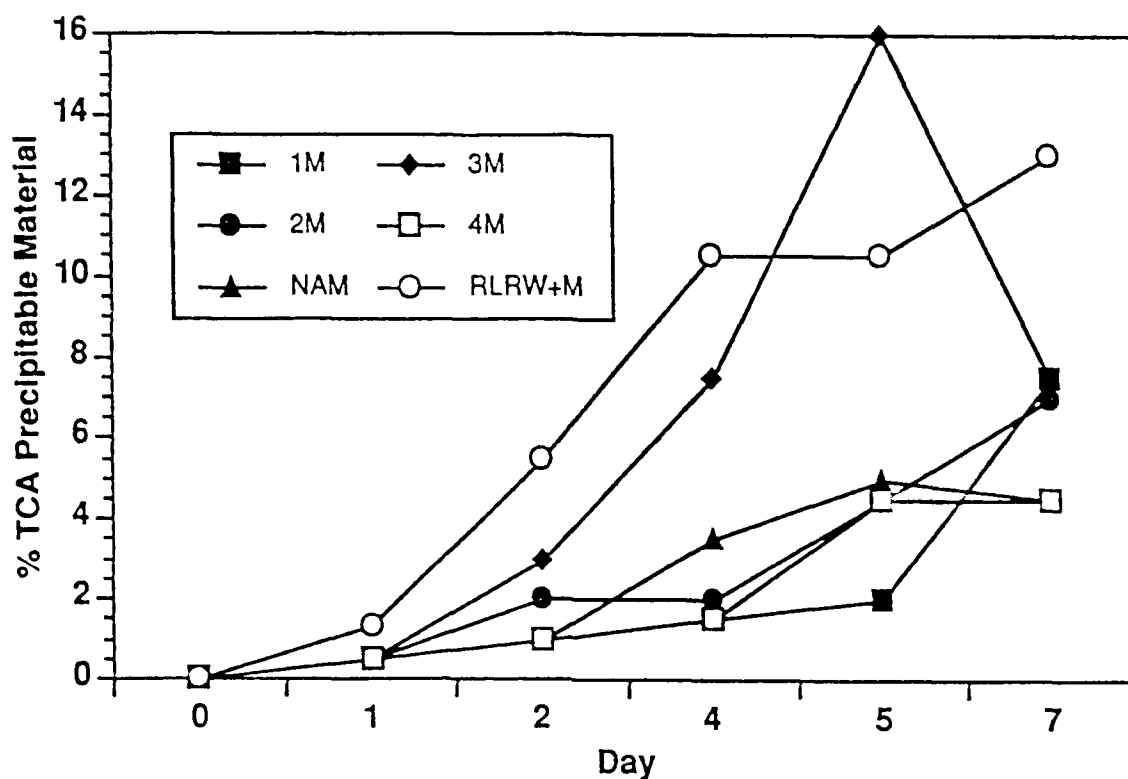


FIGURE 4 Radiolabeled TNT in TCA-Precipitable Biomass for Consortium Growing with Maleic Acid Co-Substrate

1M - Isolate #1 with 0.5% maleic acid grown at 30°C.

2M - Isolate #2 with 0.5% maleic acid grown at 30°C.

3M - Isolate #3 with 0.5% maleic acid grown at 30°C.

4M - Isolate #4 with 0.5% maleic acid grown at 30°C.

NAM - Newport consortium with 0.5% maleic acid grown at 30°C.

RLRW + M - RLRW consortium from Joliet with 0.5% maleic acid grown at 30°C.

3.4 Soil Slurry Reactor

The three slurry reactors initially established each contained NAAP soil (10% by weight). The control reactor had only NAAP soil and no additives and ran anaerobically. The second reactor had succinate (1,000 mg/L) as a co-substrate and nitrate nitrogen (100 mg/L) to initiate anoxic conditions. The third reactor had one of the more successful JAAP slurries with NAAP soil (10% by weight), succinate (1,000 mg/L), and nitrate nitrogen (100 mg/L). All reactors were purged with nitrogen initially and upon sampling. The TNT level was very high in all three reactors, at approximately a 10,000 mg/L of slurry. The reactors were sampled at 4, 8, 11, and 14 d. No removal of TNT was evident. (TNT was analyzed by using the method detailed in Appendix B.)

A second series of four slurry reactors was established with a lower initial concentration of TNT and two different co-substrates, succinate and malate. Each slurry reactor contained NAAP soil (1% by weight) yielding initial TNT concentrations in the slurry of 670 and 850 mg/L. Reactors were run with succinate (5,000 mg/L) and malate (5,000 mg/L) under aerobic and anaerobic conditions. The aerobic reactors had added ammonia nitrogen (10 mg/L), while the anaerobic reactors had added nitrate nitrogen (100 mg/L) to initiate anoxic conditions.

Sampling of these reactors at 2, 7, 10, and 14 d revealed no appreciable reduction in the TNT concentration. On the basis of these results, a hypothesis was proposed that the NAAP soil did not develop a biological population sufficiently large or diverse to reduce the TNT concentrations over the 14-d observation period because the TPH levels in the soils had a negative impact on the development of microbial flora that could use TNT as a carbon source. To test this assumption, the reactors were inoculated on day 23 with 40 mL of a JAAP TNT-acclimated slurry and 10 mL of a TPH-acclimated slurry (enriched from a soil at Sandia National Laboratories that is contaminated with diesel fuel). After the addition of approximately 50 mL of tap water, the final reactor volume was 300 mL. The solids concentration increased from approximately 1% (by weight) before the addition of the acclimated slurry to 4% (by weight) after the addition. No control for volatilization of TPH was conducted because the soils were mixed for 23 d. Any volatile components of TPH would be expected to be removed in this time period. In a reactor operated as a control, mixed and aerated with no co-substrate addition, no TNT was removed.

The reactors were analyzed on day 23 after the addition of acclimated slurry, then again on day 35. The results of this experiment, summarized in Table 4, clearly indicate that TNT can be removed from NAAP soils, even in the presence of TPH. TNT was removed in significant quantities from these reactors. These findings, in combination with the results from the work at JAAP,⁴ provide clear evidence that microbial degradation can be an effective treatment alternative for explosives-contaminated soils at NAAP.

TABLE 4 Impact of Succinate and Malate on TNT Removal from NAAP Soils in Slurry Reactors

Malate TNT (days)	Aerobic-Succinate		Anoxic-Succinate		Aerobic-Malate		Anoxic-Malate	
	TNT (mg/L)	%TNT Removed	TNT (mg/L)	%TNT Removed	TNT (mg/L)	%TNT Removed	TNT (mg/L)	%TNT Removed
0	749	0	810	0	814	0	730	0
2	722	4	731	10	738	9	771	0
7	684	9	749	8	724	11	659	10
10	763	0	709	12	694	15	867	0
14	938	0	1901	0	756	7	826	0
23	486	35	923	0	199	76	909	0
35	133	82	433	47	108	87	350	52
(Replicate Reactors)								
0	817	0	846	0	849	0	673	0
2	651	20	1106	0	643	24	695	0
7	689	16	1988	0	754	11	747	0
10	799	2	873	0	836	1	778	0
14	1106	0	1138	0	914	0	754	0
23	683	16	549	35	327	62	231	66
35	177	82	422	50	84	90	383	43
0	749	0	810	0	814	0	730	0
2	722	4	731	10	738	9	771	0
7	684	9	749	8	724	11	659	10
10	763	0	709	12	694	15	867	0
14	938	0	1901	0	756	7	826	0
23	486	35	923	0	199	76	909	0
35	133	82	433	47	108	87	350	52

4 TNT Degradation in Groundwater

Laboratory studies were conducted to determine the feasibility of developing design and operating procedures for treatment of contaminated NAAP lagoon or pond water and groundwater with a Sequencing Batch Biofilm Reactor (SBBR).

The SBBR is a fixed-film system that uses silicone tubing both to support a biofilm and to supply oxygen. Two alternative SBBR systems were investigated. One tested the ability of the white rot fungus to degrade TNT. The other used a bacterial based consortium as the inoculum and toluene as the feed to develop preliminary design and operating procedures for the SBBR. (This reactor is detailed in Appendix A.)

4.1 Removal of TNT by White Rot Fungus

Before the breakdown of TNT by the white rot fungus, *Phanerochaete chrysosporium*, was investigated in a fixed-film reactor, the activity of pure ligninase on TNT was tested. A test was run with a buffer, hydrogen peroxide, and TNT at 100 g/L. Ligninase was added to one of two flasks but not to the other (the control). After 1.5 h, the TNT in the flask that contained ligninase was reduced by 32% as compared to the control.

A fixed-film reactor was established for growing white rot fungus. After the fungus reached secondary metabolism, TNT was added to the reactor at an initial concentration of 83 mg/L. Samples collected every 3 d for 15 d showed that the concentration of TNT in the reactor decreased to 1.9 mg/L, with most of the TNT removed during the first 3 d. This preliminary evaluation of the white rot fungus system was followed by three additional experiments. In all tests, *P. chrysosporium* (BKM.F-1767) was immobilized on the silicone tubing placed in a SBBR with 2-L liquid volume. At predetermined times during immobilization, the medium used for growth was replaced by a production medium that facilitates production of lignin peroxidases that are known to catalyze the initial oxidation of a variety of organopollutants, including TNT and RDX.

In the first experiment, 60 mg of TNT in 1 mL of chloroform was added to a 13-day-old system in which the growth medium had been changed to production medium 10 d before TNT was added. After 50 h of incubation, only about 5% of the TNT added could be recovered in the extracellular fluid (Fig. 5). Extraction of the mycelium with chloroform

and subsequent analysis of the chloroform extract for TNT showed that adsorption by the mycelium was not a significant cause for the TNT disappearance observed. In the second experiment, 60 mg of TNT in 1 mL of acetone was added to a 14-day-old system in which the growth medium was changed to production medium 11 d before the TNT was added. The use of acetone instead of chloroform as the addition solvent apparently solved a TNT solubility problem encountered in the first experiment. Approximately 83% of the TNT added was degraded during the first 72 h (Fig. 6). In these two experiments, neither culture developed was ligninolytic (i.e., lignin peroxidase activity was not expressed).

Biodegradation of TNT in a third ligninolytic culture (30.7 units of veratryl alcohol oxidase activity per liter) is presented in Fig. 7. In this experiment, 66 mg of TNT was added to an 8-day-old system in 7.95 mL of 10:1 methanol:acetone. Initially *P. chrysosporium* was incubated in growth medium in the bioreactor. After 4 d, the growth medium was changed to production medium. After 3 d the bioreactor was drained, and fresh production medium was added. After another 24 h, TNT was added. The results shown in Fig. 7 demonstrate that extensive biodegradation of TNT occurred. After 96 h of incubation, TNT was not detected in the culture medium, even after the amount of material routinely used for analysis was increased eight fold.

The above results demonstrate that TNT disappeared in SBBRs inoculated with *P. chrysosporium*. Interestingly, the removal of TNT occurred in the first two experiments when lignin peroxidase activity was not being expressed. This is of significance because the lignin-degrading system of *P. chrysosporium* is thought to be responsible, at least in part, for the ability of the organism to degrade a wide variety of structurally diverse organopollutants. Because of these considerations, experiments were initiated to determine why cultures in the bioreactor did not become ligninolytic. Results showed that maintenance of pH was critical. When the pH of the production medium was maintained at 4.5 by the addition of HCl, *P. chrysosporium* immobilized on silicone tubing in the bioreactor became ligninolytic (30-275 units/L of veratryl alcohol oxidase activity).

The biodegradation of TNT under nonligninolytic conditions is of considerable interest because it indicates that TNT can be used under both nutrient-rich and nutrient-limited conditions. In all analyses, TNT was the only chemical that was accounted for.

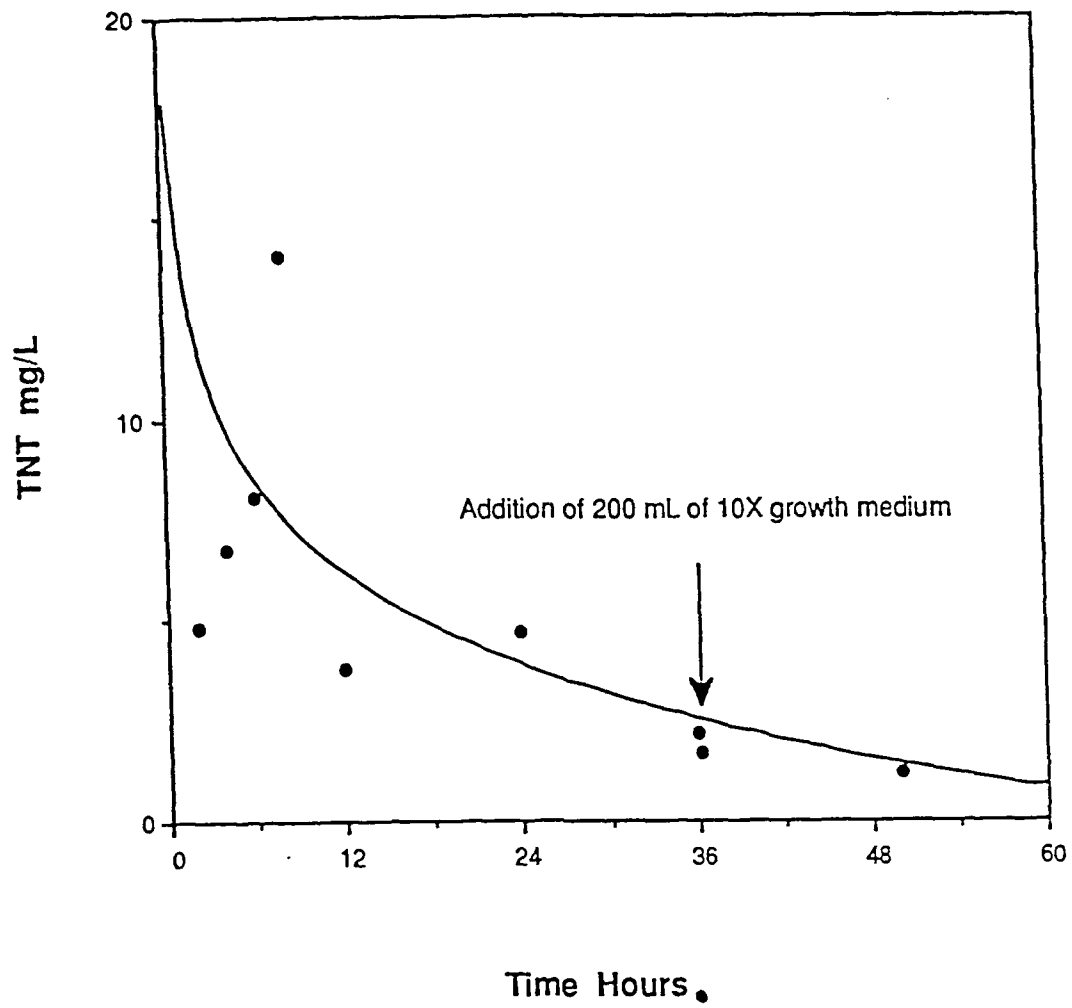


FIGURE 5 Biodegradation of TNT by *P. chrysosporium* in SBBR Experiment 1

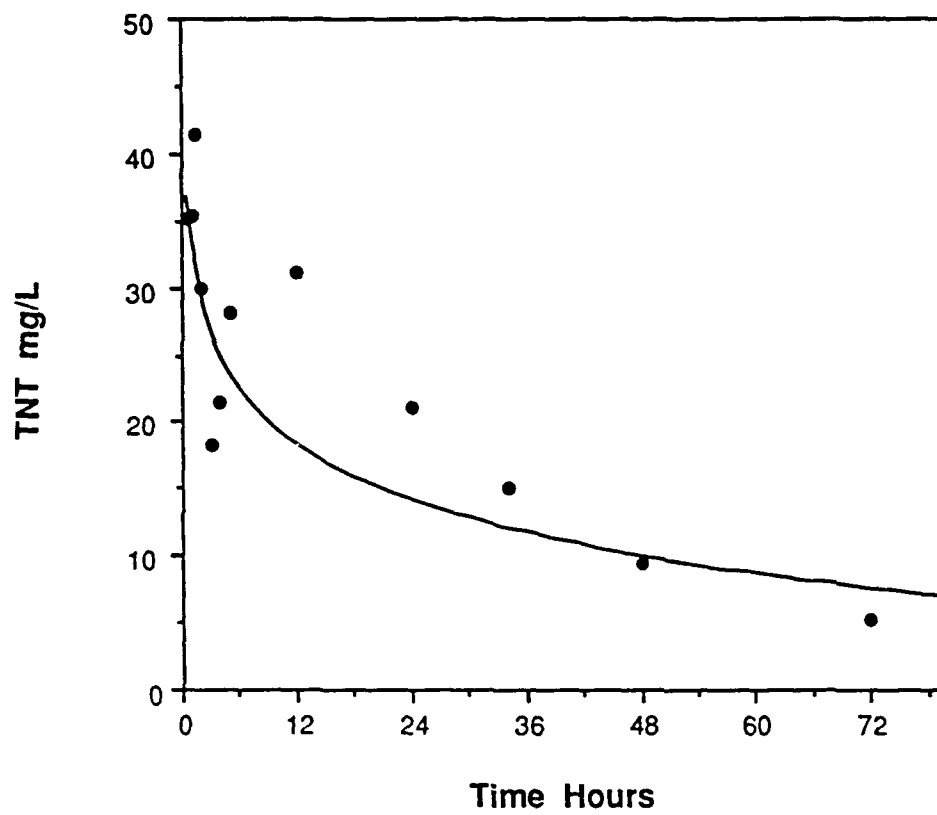


FIGURE 6 Biodegradation of TNT by *P. chrysosporium* in SBBR Experiment 2

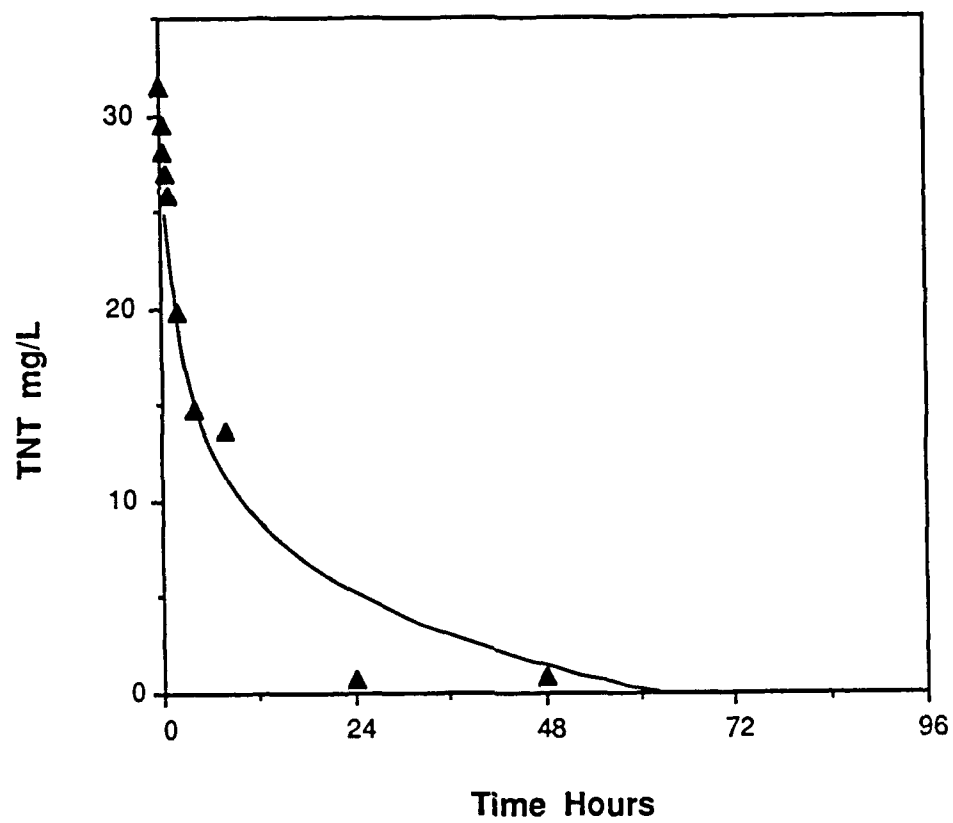


FIGURE 7 Biodegradation of TNT by *P. chrysosporium* in SBBR Experiment 3

These experiments, although promising, have several aspects that may make pilot scale application very difficult. A pure fungal culture of *P. chrysosporium* was used with a waste stream containing only TNT and no other organic materials. Contaminated groundwater generally contains many different indigenous organisms, which may be expected to compete for substrate, oxygen, and surface area on the fixed-film reactor. In treating groundwater, several other potential organic substrates that could be present may be preferentially used by *P. chrysosporium*. Finally, *P. chrysosporium* is currently regulated by the U.S. Department of Agriculture as a potential plant pathogen. Thus, significant regulatory hurdles exist in its use in environmental work.

4.2 Operating Information for a Bacterial Based SBBR

To investigate methods of treating groundwater that may be contaminated with organic compounds, a novel reactor configuration was examined. This system was intended to treat groundwater contaminated with organics such as toluene and benzene.

The SBBR was initially seeded with *Pseudomonas putida* mt-2. This organism contains a plasmid, designated TOL, that contains the genetic information necessary to produce the enzymes for the degradation of toluene and related compounds (e.g., benzene, ethylbenzene, and xylenes). This particular organism degrades toluene by first oxidizing the methyl group to a carboxylic acid group, then converting benzoic acid to catechol, which is cleaved by a 2,3-catechol dioxygenase to form 2-hydroxymuconic semialdehyde. This compound is then directed to the Krebs cycle, where it is completely oxidized to CO₂ and H₂O. In this preliminary study, the focus was on design and operating strategies for a bench scale SBBR. To this end, a synthetic waste stream containing toluene was used as the carbon and energy source.

For the first four weeks of operation, the feed was mixed daily by placing 2 L of nutrient medium into a flask and adding 0.5 mL of toluene. The flask was then sealed and mixed overnight. When the feed was prepared in this manner, the toluene concentration introduced to the reactor was approximately 100-130 mg/L. For the next eight weeks of operation, the feed was mixed in a 20-L bottle by adding excess volume (approximately 20 mL) to the nutrient medium and allowing the solution to mix continuously. Feed for the reactor was removed from the bottom of the jar so that no free toluene was introduced to the reactor. During this time, the toluene concentration in the feed was 170-200 mg/L.

The reactor, a 4-L glass kettle, was operated in a sequencing batch mode with a total cycle time of 12 h. The cycle began with a 1-h fill period, during which 1 L of synthetic waste water was introduced into the reactor. This period was followed by a 10-h react period and then a 1-h draw period, during which 1 L of treated effluent was removed from the reactor. Oxygen supply to the silicone tubing continued for the entire operating cycle. The oxygen pressure in the silicone tubing was varied between 1 and 10 psig to determine the impact on performance. Since the reactor was designed as a biofilm system rather than a suspended growth system, no settling period was needed as is typically the case for sequencing batch reactors. Furthermore, at the completion of a cycle, the next cycle began immediately, eliminating the idle period from the operation of this reactor. The high water level in the reactor was 3 L, and the low water level was 2 L. Two cycles were completed each day, giving the reactor a 1.5-d hydraulic retention time. Since a 4-L kettle was used, the headspace in the reactor was approximately 1 L. The reactor was sealed except for a carbon trap that collected any toluene that volatilized into the headspace and was displaced from the system during filling.

The concentration of toluene in the effluent during the 3-mo study was less than 10 $\mu\text{g/L}$ when the oxygen pressure in the silicone tubing was sufficiently high to meet the demand for oxygen and the biofilm thickness was not too great. Figure 8 shows toluene profiles taken before and after the silicone tubing was removed from the reactor and the biofilm was washed with a stream of water. The biofilm was several millimeters thick at the time of removal. The quantity of toluene recovered from the activated carbon traps placed on the reactor (i.e., the measure of the loss of toluene by volatilization from the SBBR) was negligible.

These results demonstrate that the SBBR is an effective system for treating waste waters containing volatile, biodegradable compounds if large quantities of biomass are not allowed to accumulate on the silicone tubing. If some of the original water-soluble contaminants or intermediates formed at NAAP are nonbiodegradable, granular activated carbon (GAC) may have to be added to the SBBR system. To test the performance of such a system, a second SBBR was operated for 30 weeks with GAC. The performance of this GAC-SBBR equalled that of the SBBR. At the end of the study, the activated carbon bed was thoroughly mixed and extracted to determine the quantity of toluene adsorbed. Duplicate samples revealed that 23 mg of toluene were adsorbed for each gram of activated carbon in the reactor, or approximately 10% of the total mass of toluene fed to the reactor.

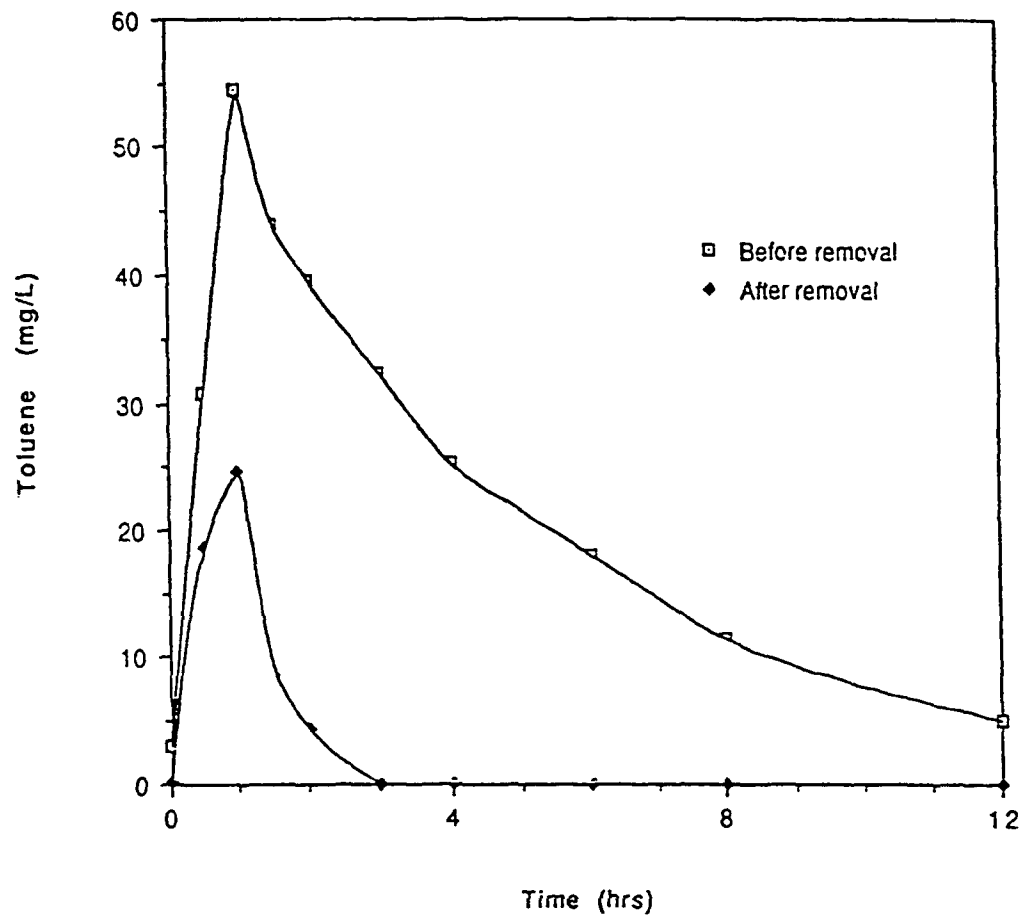


FIGURE 8 Toluene Concentration Profiles before and after Removal of Biomass

5 Conceptual Treatment System for Soil and Water

Results to date demonstrate the feasibility of bioremediation of the NAAP soils. Bioremediation can be accomplished in an SS-SBR like the one being developed for similar soils at the JAAP site. The SS-SBR is an equipment- and labor-intensive solution that will achieve treatment in a relatively short time and in a small land area.

5.1 The SS-SBR

The SS-SBRs at NAAP could be designed to be operated in a sequencing batch mode, like those currently being designed for the JAAP bioreactor demonstration project. In the JAAP system, each cycle of SS-SBR operation involves three discrete periods, *FILL*, *REACT*, and *DRAW*.

During *FILL*, a contaminated soil slurry and any makeup water that is needed to achieve the proper solids concentration (typically 10-20% by weight) are added to a tank that is being mixed. The volume added depends on the percent replacement that has been established for a given tank. For example, a total of 17.5 gal (5% of 350 gal, the usable volume of each tank proposed for JAAP) will be added to a tank during each cycle if it is being operated as a 5% replacement system. After *FILL*, a chemical feed system will deliver the required amounts of nutrients, co-substrate, and pH-adjusting chemicals.

The *REACT* period follows *FILL*. During *REACT*, the mixers remain on, and the reactions necessary to degrade the explosives take place. When oxygen serves as the exogenous electron acceptor, the aeration system is activated. If nitrate were to be the exogenous electron acceptor, only the mixing system would be used to suspend the slurry. In either case, the co-substrate will serve as the primary carbon and energy source, and the soil contaminants TNT, RDX, and HMX will be co-metabolized. The time provided for *REACT* will be dictated by the rate of utilization of the target compounds. The results obtained from the bench scale studies indicate that the time for *REACT* will be 11 h if the system operates with two cycles per day or 23 h if the system operates with one cycle per day.

After *REACT*, a mixed, treated slurry is removed from the tank during *DRAW*. The volume withdrawn will equal the volume added during *FILL*. The total time for each

cycle will be either 12 or 24 h. After DRAW, the tank will be refilled and the entire process repeated.

5.2 Groundwater Treatment with an SBBR

As discussed previously, insufficient information is available to design and construct a field SBBR unit for treating the explosives-contaminated groundwater at NAAP. The development of a complete program for an SBBR pilot facility at NAAP would require additional bench scale studies. These studies would require the use of groundwater and/or lagoon water from NAAP that is contaminated with TNT and other nitro-based contaminants and intermediates generated during SS-SBR operation. These studies would need to develop information regarding acceptable variations in contaminant loading, the degree of contaminant destruction attainable with various process throughput rates, and the long-term viability of the biological system.

6 Conclusions and Recommendations

Results to date indicate that the soils at the NAAP can be remediated with an SS-SBR. Aerobic, anoxic, and anaerobic organisms are present at the site. Slurry reactors inoculated with acclimated NAAP slurries achieved considerable TNT removal even in the presence of high concentrations of TPH. Organisms needed to degrade 2,4-DNT and 2,6-DNT are available. SBBRs inoculated with *P. chrysosporium* demonstrated that TNT is readily degraded by this white rot fungus, even when the organism is not ligninolytic. A bacterial based SBBR was operated successfully at the bench scale. The data obtained can be used to design and operate a pilot scale SBBR efficiently. These results, coupled with the improved enrichments and the accelerated rates associated with the use of various co-substrates in the biodegradation studies, clearly indicate that the treatment of the explosives-contaminated soil at NAAP with a bacterial based consortium is feasible and that additional laboratory scale tests are warranted.

A review of the draft remedial investigation report being prepared by Dames and Moore indicates that the vast majority of the contaminated soils present at NAAP have low TPH concentrations and that most contain TNT concentrations much less than those found in the samples evaluated. In addition, organisms capable of degrading both 2,4-DNT and 2,6-DNT are present at the site. This information and experimental experience with TNT-contaminated soils from JAAP indicate that bioremediation using a bacterial based consortium is a promising treatment alternative, especially if soils contaminated with high concentrations of TPH are blended with soils with low TPH concentrations or if a physical or chemical method is used to remove the hydrocarbons before bioremediation.

The laboratory work indicates that the biological methods examined for treating explosives-contaminated groundwater show great promise as a cost-effective treatment alternative. However, at least one more series of laboratory experiments is necessary before these systems should be considered for treatability studies. In particular, information must be obtained regarding the stability of the microbe populations when they are exposed over a long period of time to the microbial community resident in the groundwater. Additional studies must also be performed to gather further information about the effect of large swings in the contaminant concentration on the performance of the described groundwater treatment systems.

Costs associated with the treatment of the contaminated soils can be expected to mirror the process costs established in the JAAP feasibility study,⁴ estimated at \$100-130 per cubic yard. Treatment costs for the explosives-contaminated groundwater cannot be estimated with any reasonable certainty at this time. Additional bench scale experiments must be performed to develop the design information necessary to prepare a credible cost estimate.

7 References

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Appendix A

Laboratory Reactor Schematic Drawings

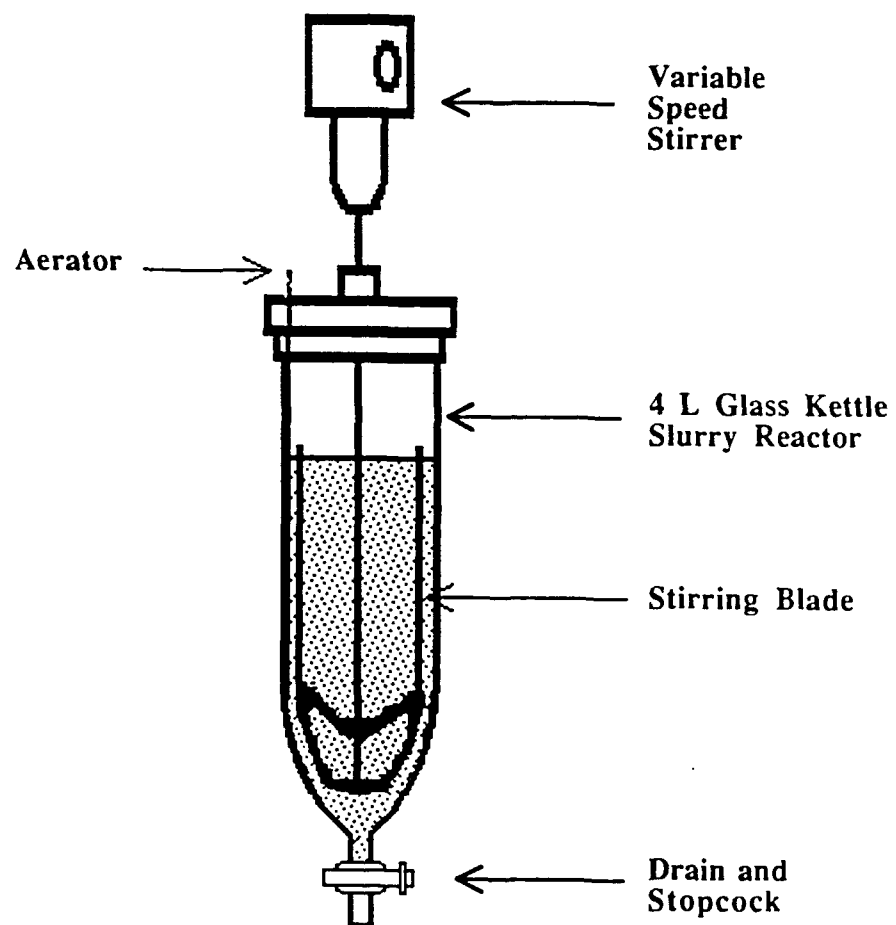


FIGURE A.1 Soil Slurry Sequencing Batch Reactor

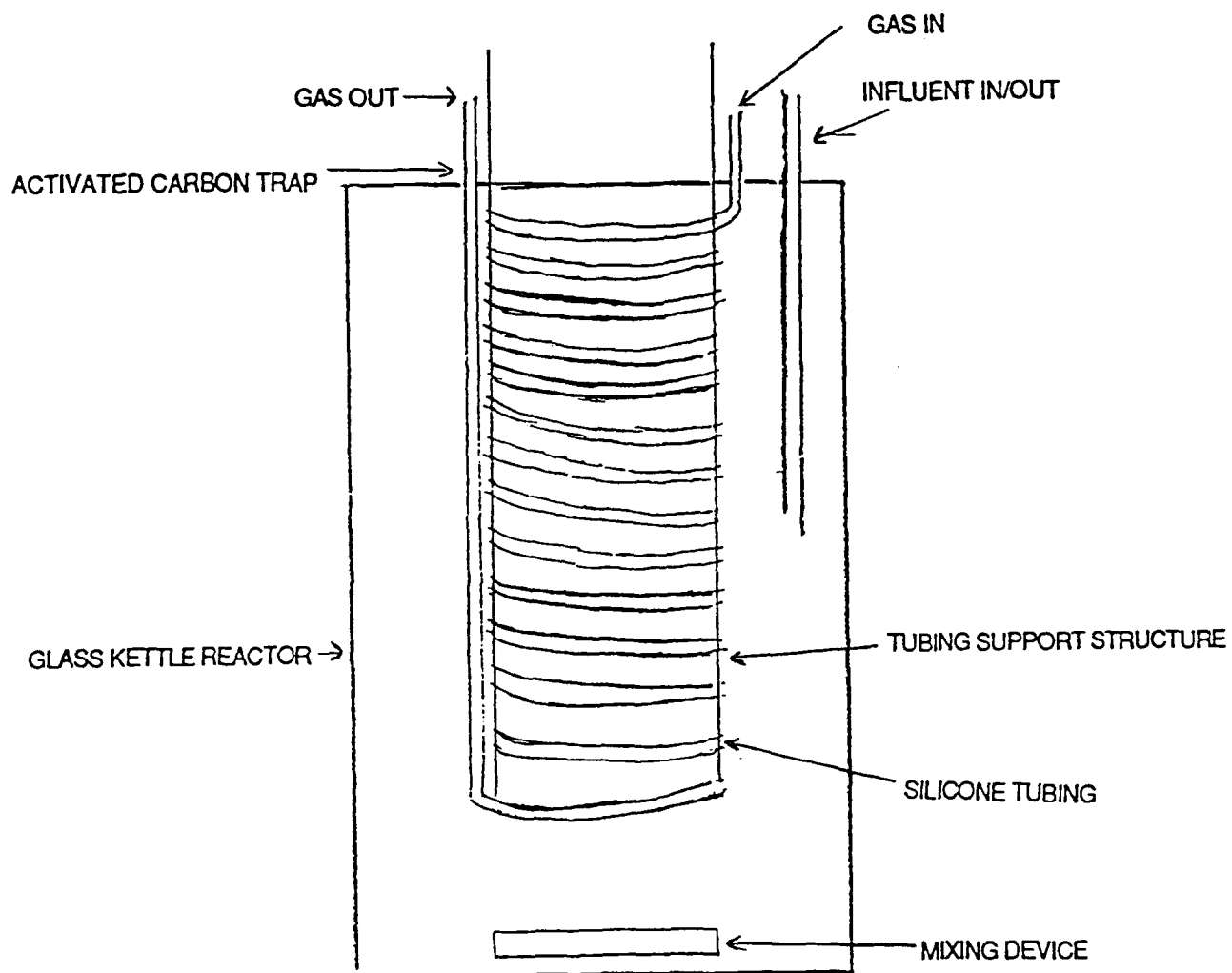


FIGURE A.2 Silicone Tubing Reactor

Appendix B
Explosives Analysis Procedure

EXPLOSIVES IN SOIL

I. SUMMARY

A. Analytes:

HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
RDX	Hexahydro-1,3,5-trinitro-s-triazine
NB	Nitrobenzene
1,3-DNB	1,3-Dinitrobenzene
1,3,5-TNB	1,3,5-Trinitrobenzene
2,4-DNT	2,4-Dinitrotoluene
2,6-DNT	2,6-Dinitrotoluene
2,4,6-TNT	2,4,6-Trinitrotoluene
Tetryl	2,4,6-Trinitrophenylmethylnitramine

B. Matrix: Soil or sediment

C. General Method: An aliquot of soil is extracted with acetonitrile. The acetonitrile is diluted with methanol and water, and the resultant solution is injected onto the HPLC for analysis.

II. APPLICATION

A. Tested Concentration Range:

HMX	1.27-140 ug/g
RDX	0.98- 80.0 ug/g
NB	0.42- 60.0 ug/g
1,3-DNB	0.59- 60.0 ug/g
1,3,5-TNB	2.09- 60.0 ug/g
2,4-DNT	0.42- 60.0 ug/g
2,6-DNT	0.40- 60.0 ug/g
2,4,6-TNT	1.92-100.0 ug/g
Tetryl	0.32- 24.9 ug/g

B. Sensitivity:

Peak Height in mm at an Attenuation of 2^4

HMX	48 mm for 14 ug/g
RDX	48 mm for 8.0 ug/g
NB	26 mm for 6.0 ug/g
1,3-DNB	53 mm for 6.0 ug/g
1,3,5-TNB	44 mm for 6.0 ug/g
2,4-DNT	31 mm for 10.0 ug/g
2,6-DNT	17 mm for 6.0 ug/g
2,4,6-TNT	45 mm for 6.0 ug/g
Tetryl	26 mm for 8.0 ug/g

C. Detection Limits:

HMX	1.27 ug/g
RDX	0.98 ug/g
NB	0.42 ug/g
1,3-DNB	0.59 ug/g
1,3,5-TNB	2.09 ug/g
2,4-DNT	0.42 ug/g
2,6-DNT	0.40 ug/g
2,4,6-TNT	1.92 ug/g
Tetryl	0.32 ug/g

D. Interferences:

1. Any compound that is extracted from soil that gives a retention time similar to the nitro-compounds and absorbs at 250 nm.

E. Analysis Rate:

After instrument calibration, one analyst can analyze two samples in one hour. One analyst can conduct sample preparation at a rate of three samples per hour. One analyst doing both sample preparation and the HPLC analysis can run 16 samples in an 8-hour day.

F. Safety information:

Work in well-ventilated areas. Wear adequate protective clothing to avoid skin contact. Wash skin with soap and water thoroughly immediately after contact.

TNB, HMX, RDX, Tetryl, and TNT's are classified as Explosives A by DOT. Avoid extreme temperatures and pressures.

III. APPARATUS AND CHEMICALS

A. Glassware/Hardware

1. Syringes: 10 μ L, 50 μ L, 100 μ L, 1 mL syringe (Hamilton 1005 TEFLL)
2. Vials with Teflon-lined caps or septa. Nominal volume of 1.8 mL, 4.0 mL and 8.0 mL.
3. B-D Glaspak disposable syringes, 5 mLs, with frosted tip
4. 0.2 micron fluorocarbon filters
5. Micropipettes, 200 μ L
6. Hypo needles
7. 2 mL. pipette

B. Instrumentation

1. Perkin-Elmer Series 4 High Performance Liquid Chromatograph (HPLC) equipped with a Perkin-Elmer ISS100 Auto-Injector and Micromeritrics Model 786 UV/VIS variable wavelength detector. Hewlett-Packard 3390 recording integrator in peak height mode was used to record data output. ISS 100 auto injector is equipped with a temperature controlled sample tray jto refrigerate extracts.

2. Analytical Balance

Capable of weighing 0.01 grams for sample preparation and 0.1 mg for standard preparation. Mettler AE 163 or equivalent.

3. Parameters

a. Columns:

- 1) DuPont Zorbax^R ODS 4.6 mm i.d. x 25 cm HPLC column with a particle size of 5-6 microns.
- 2) DuPont Permaphase^R ODS guard column. (optical)

- b. Mobile Phase: The water/methanol ratio must be adjusted as described in the calibration Section V.A.5.c to obtain optimum peak separation.

44-55% water
28-34% methanol
16-22% acetonitrile

- c. Flow: 1.6 mL/min with a pressure of approximately 2860 psig.
d. Detector: 250 nm
e. Injection Volume: 50 uL
f. Retention Times:

	<u>Minutes</u>
HMX	3.30- 3.60
RDX	4.55- 4.70
NB	7.95- 9.00
1,3-DNB	7.30- 8.00
1,3,5-TNB	6.35- 6.40
2,4-DNT	11.00-13.10
2,6-DNT	10.60-12.40
2,4,6-TNT	10.05-10.90
Tetryl	9.15- 9.70

C. Analytes

1. Chemical Abstracts Registry Numbers

HMX	2691-41-0
RDX	121-82-4
NB	98-95-3
1,3-DNB	99-65-01
1,3,5-TNB	99-35-4
2,4-DNT	121-14-2
2,6-DNT	606-20-2
2,4,6-TNT	118-96-7
Tetryl	35572-78-2

2. Chemical Reactions

- a. RDX and HMX can undergo alkaline hydrolysis.
b. RDX and HMX degrade at temperatures greater than 80°C in an organic solvent.

3. Physical Properties

	<u>Formula</u>	<u>Mol. Wt.</u>	<u>M.P. (°C)</u>	<u>B.P. (°C)</u>
HMX	$C_4H_8N_8O_8$	296.6	276	-
RDX	$C_3H_6N_6O_6$	222.12	205	-
NB	$C_6H_5NO_2$	123.11	6	211
1,3-DNB	$C_6H_4N_2O_4$	168.11	90	302
1,3,5-TNB	$C_6H_3N_3O_6$	213.11	122	315
2,4-DNT	$C_7H_6N_2O_4$	182.14	71	300 (decomposes)
2,6-DNT	$C_7H_6N_2O_4$	182.14	66	-
2,4,6-TNT	$C_7H_5N_3O_6$	227.13	82	240 (decomposes)
Tetryl	$C_7H_5N_5O_8$	287.15	131	157

D. Reagents and SARMS:

1. Acetonitrile, distilled in glass for HPLC use
2. Methanol, distilled in glass for HPLC use
3. Water, distilled in glass for HPLC use
4. USATHAMA Standard Soil

5. SARMS

HMX	SARM No.	1217 (PA 1303)
RDX	SARM No.	1130 (PA 1302)
NB	SARM No.	(PA 1306)
1,3-DNB	SARM No.	2250 (PA 1305)
1,3,5-TNB	SARM No.	1154 (PA 1300)
2,4-DNT	SARM No.	1147 (PA 1298)
2,6-DNT	SARM No.	1148 (PA 1299)
2,4,6-TNT	SARM No.	1129 (PA 1297)
Tetryl	SARM No.	1149 (PA 1301)

IV. CALIBRATION

A. Initial Calibration

1. Preparation of Standards:

a. Stock calibration solutions containing approximately 10,000 mg/L of a nitro-compound are prepared by accurately weighing ca. 50 mg of a SARV into a 5 mL serum bottle and dissolving the nitro-compound in 5 mL of acetonitrile pipetted into the bottle. All stock solutions prepared in this manner and stored in a freezer (0°C to -4°C) have remained stable for a period of 6 months.

b. Intermediate Calibration Standards: All compounds appear to be stable for at least 3 months.

1) Intermediate Calibration Standard A (high level): Combine the appropriate volumes of stock calibration standard as shown below. Dilute to 5 mL with acetonitrile and seal with a Teflon-lined cap. Store in the dark at 0°C-4°C. The resulting solution will have the concentrations indicated in the following table.

<u>Nitro-compound</u>	<u>uL of Stock Cal Std</u>	<u>Resulting concentration (ug/mL)</u>
FMX	175	350
RDX	100	200
NB	75	150
1,3-DNB	75	150
1,3,5-TNB	75	150
2,4-DNT	75	150
2,6-DNT	75	150
2,4,6-TNT	125	250
Tetryl	100	200

- 2) Intermediate Calibration Standard B (low level): 1:10 dilution of the Intermediate Calibration Standard A is made in Acetonitrile. Seal with a Teflon-lined cap and store in the dark at 0°-4° C. The resulting solution will have the following concentrations:

<u>Nitro-Compound</u>	<u>Resulting conc. (ug/mL)</u>
FMX	35.0
POX	20.0
NS	15.0
1,3-DNB	15.0
1,3,5-TNB	15.0
2,4-DNT	15.0
2,6-DNT	15.0
2,4,6-TNT	25.0
Tetryl	20.0

- c. Working Calibration Standards: Using the following table, prepare a series of ten calibration standards. Place the mobile phase into a 1-mL serum vial. Inject the indicated volumes of intermediate calibration standard A or B into the acetonitrile with a microliter syringe. Seal the vial with a teflon-lined septum and cap. Mix well. These solutions are prepared fresh daily and kept in the dark.

WORKING CALIBRATION STANDARDS

Conc.	Amt. (uL) Intermed. Cal. Std. to Add		Amt. (uL) Mobile Phase to Add	Resulting Concentration (ug/L)			
	A	B		FMX	2,4,6-TNT	Tetryl POX	1,3-DNB 1,3,5-TNB 2,6-DNT 2,4-DNT
0	0	0	2.0	-	-	-	-
0.2 X	-	1.0	999.0	35	25	20	15
0.5 X	-	2.5	997.5	87.5	62.5	50	37.5
1 X	-	5	995.0	175	125	100	75
2 X	-	10	990.0	350	250	200	150
5 X	-	25	975.0	875	625	500	375
10 X	5	-	995.0	1750	1250	1000	750
20 X	10	-	990.0	3500	2500	2500	1500
50 X	25	-	975.0	8750	6250	5000	3750
100 X	50	-	950.0	17500	12500	10000	7500

2. Instrument Calibration

- a. Set up the instrument according to the manufacturer's recommendations.
- b. Mobile Phase is analyzed as a blank to verify a stable baseline.
- c. Analyze the medium calibration standard (10X) to verify peak separation and retention times.
- d. Analyze the calibration standards prepared in Section IV.A.1.

3. Analysis of Calibration Data

- a. Tabulate the calibration standard concentration versus the peak height response for each calibration standard.
- b. Perform a linear regression analysis on the calibration data plotting peak height vs. concentration in $\mu\text{g/l}$.

4. Calibration Checks

- a. After completion of analyses of samples, a calibration standard at the highest concentration is analyzed. The response must agree within 25% for that concentration from the first seven calibration curves. Thereafter, the response must agree within two standard deviations of the mean response for that concentration. If it does not, the calibration standard will be reanalyzed. If the calibration standard fails this test, initial calibration must be performed, and all samples analyzed since the last acceptable calibration must be reanalyzed.
- b. No certified calibration check standards are available for these compounds.

B. Daily Calibration

1. Prior to analyses each day, a high calibration standard will be analyzed. For the first seven determinations at this concentration, the response must agree within 25% of the mean of all previous responses. After seven determinations, the response must agree within \pm two standard deviations of the mean response for previous determinations at this concentration.

2. If the calibration standard fails this test, it will be reanalyzed. If the calibration standard fails the second test, the system will have failed daily calibration, and initial calibration will be performed.
3. After completion of sample analyses each day, the high calibration standard will be analyzed again. The response for this calibration standard will be subjected to the criteria discussed in Section IV.B.1, above. If the response fails the criteria, the standard will be reanalyzed. If the second response fails the test, the system will have failed calibration, and initial calibration will be performed. All samples analyzed since the last acceptable calibration must be reanalyzed.

V. Certification Testing

A. Control Spikes:

To a series of ten 5-mL serum vials, approximately one gram of soil is accurately weighed into each vial. Using a syringe, the volumes of intermediate calibration standard indicated in the following table are injected onto the soil. The serum vial is covered with a septum and shaken until the soil no longer looks wet (approximately 60 seconds). The sample must equilibrate at least one hour. The septum is removed and the indicated amount (see Table below) of acetonitrile is pipetted onto the soil. The septum is replaced and the vial is capped. The sealed sample is shaken by hand for approximately 2-3 minutes. The sample is prepared via the procedure given in this method, to give the target concentrations in the following table.

CONTROL SPIKES

Resulting Concentration (ug/g)

Conc.	Amt. (uL) Intermed. Cal. Std. to Add		Amt. (uL) Aceto Nitrile to Add	FMX	2,4,6 TNT	Tetryl RDX	1,3-DNB
	A	B					1,3,5-DNB
							2,6-DNT
							2,6-DNT NB
0	0	0	2000	0	0	0	0
0.2 X	-	8.0	1992	0.28	0.2	0.16	0.12
0.5 X	-	20	1980	0.70	0.5	0.4	0.3
1 X	4	-	1996	1.40	1.0	0.8	0.6
2 X	8	-	1992	2.80	2.0	1.6	1.2
5 X	20	-	1980	7.0	5.0	4.0	3.0
10 X	40	-	1960	14.0	10.0	8.0	6.0
20 X	80	-	1920	28.0	20.0	16.0	12.0
50 X	200	-	1800	70.0	50.0	40.0	30.0
100 X	400	-	1600	140.0	100.0	80.0	60.0

VI. SAMPLE HANDLING STORAGE

- A. Sampling Procedure: The stability of explosives in soil is not truly known. Precautions should be taken to avoid prolonged exposure to light and heat.
- B. Containers: Wide-mouth amber glass bottles with teflon-lined lids.
- C. Storage Conditions: Samples should be maintained at 4°C from the time of collection to the time of analysis. No chemical preservatives are necessary.
- D. Holding Time Limits: 7 days to extraction; 40 days to analysis from the time of extraction.
- E. Solution Verification: No certified check standards are available.

VII. PROCEDURE

A. Separations

1. Accurately weigh 1 gram of soil into a 5-mL serum vial and pipette 2 mL of acetonitrile onto the soil.

2. Place a septum and cap on the vial and shake the vial thoroughly by hand for 2-3 minutes.

3. The extract is then filtered using the following technique.

A 5-mL syringe is fitted with a needle. After the extract is drawn into the syringe barrel, a Fluorocarbon 0.2 micron disposable filter is attached in place of the needle. The sample is then slowly forced through the filter into a 4.0 mL teflon capped vial and stored until the extract is diluted and analyzed by HPLC. (Step 4-C.)

4. Preparation of sample extracts and spikes for injection is performed the day of analysis.

- a. Using a disposable micropipette, accurately measure 200 μ L of filtered extract into a 1-mL vial. Accurately measure 600 μ L of a 33% methanol/67% water solution onto the filtered sample. This will produce 800 μ L of extracted sample in mobile phase.

- b. Place a septum cap on the vial. Shake the vial well to thoroughly mix. Store in the dark at 0°-4° C until ready to analyze.

B. Chemical Reactions - None. Compounds are read directly.

C. Instrumental Analysis:

1. Set the chromatographic conditions as follows:

	Time (minutes)	Flow (mLs/min.)	MeOH %	MeOH %	HOH %
Equilibrium	2	1.6	16	34	50
Analysis Run	20	1.6	16	34	50

2. All standards and extracts should be in chilled tray (4° C)
3. Using the auto-injector manufacturer's recommended procedure, introduce 50 μ L of the medium level calibration standard into the

chromatographic system. Check the chromatogram to ensure separation of the nitrated toluenes and separation of the nitrobenzene and tetraol. If necessary, adjust the water/ methanol ratio of the mobile phase until separate peaks are distinguished. As the column ages, less methanol is required. Generally, the column ages rapidly the first 24 hours, after which it is fairly stable.

- 4) Once good peak separation is obtained, introduce 50 μ L of each working calibration standard and sample into the chromatographic system using the auto-injector manufacturer's recommended procedure.

VIII. CALCULATIONS

- A. The diluted extract concentration is read or calculated from the instrument calibration curve.

- B. Sample Concentration (μ g/g) = extract conc $\times \frac{B \times D}{A \times C}$

where:

A = sample weight (dry weight)

B = mL acetonitrile used to extract sample

C = mL acetonitrile extract diluted into mobile phase

D = final volume in mL of mobile phase prepared for injection

NOTE: When samples are prepared according to this method (1 gram extracted into 8 mL of mobile phase), the above calculation becomes:

$$\text{Sample Concentration } (\mu\text{g/g}) = \text{extract conc } (\mu\text{g/l}) \times 0.008$$

IX. DAILY QUALITY CONTROL

A. Control Samples

1. Intermediate Spiking Standard A and B are made according to Section IV just as calibration standards.

2. Daily control samples are prepared in a manner identical to that described in Section V. A total of three control spikes are required on a daily basis: two at 10X and one at 2X. They will have the following concentrations.

Conc.	Amt (uL) Intermed. Spiking A to add to 2.0 mls Acetonitrile					2,4-DNT 2,6-DNT 1,3-DNB 1,3,5-DNB NB	
	Conc.	FMX	2,4,6-TNT	Tetryl RDX			
2X	8	2.8	2.0	1.6		1.2	
10X	40	14.0	10.0	8.0		6.0	

3. At least one method blank using the USATFMA Standard Soil is also analyzed with each analytical lot.
4. At least one matrix spike (actual sample) at 10X is analyzed for each analytical lot or at a frequency of 10%, whichever is more frequent.

B. Control Charts:

1. Average Percent Recovery (X)

- Percent recoveries for the 10X certification spikes from days 1 and 2 are averaged to obtain the first value to be plotted.
- Percent recoveries for the 10X certification spikes from days 3 and 4 are averaged to obtain the second value to be plotted.
- Percent recoveries for the method spikes closest to the certification 10X concentration from the first day of analyses are averaged to obtain the third value to be plotted.
- Values from a, b, and c are averaged to determine the central line of the control chart.
- Differences in percent recoveries for each pair of values in a, b, and c are averaged to obtain R.
- The upper and lower warning limits are $\pm 1.25 R$ from the central line.

- g. The upper and lower control limits are $\pm 1.83 R$ from the central line.
2. Difference in percent recoveries (R)
- a. The value for R obtained in Section IX.B.1.e, above, is the base line of the control chart.
 - b. The warning limit is $2.511 R$.
 - c. The control limit is $3.267 R$.
3. Three Point Moving Average X
- a. The average percent recovery from the $5 \mu\text{g/g}$ concentration from the first three days of certification testing is the first point to be plotted.
 - b. Subsequent points to be plotted are the average percent recoveries from the $5 \mu\text{g/g}$ concentration from the next group of three determinations (e.g., certification days 2, 3, and 4; certification days 3 and 4 and the first day of analysis; certification day 4, day 1 of analysis, and day 2 of analysis; etc.)
 - c. The central point on the control chart is the average of the plotted points and changes with each added point.
 - d. The range for each point is the difference between the highest and lowest values in each group of three determinations. The average range (MAR) is used to define the warning and control limits.
 - e. The upper and lower warning limits are $\pm 0.682 \text{ MAR}$, respectively.
 - f. The upper and lower control limits are $\pm 1.023 \text{ MAR}$, respectively.
4. Three point Moving Average R:
- a. The base line is the MAR.
 - b. The warning limit is 2.050 MAR .
 - c. The control limit is 2.575 MAR .

5. Certified Calibration Check Standard:

- a. If available, two certified calibration check standards are analyzed with samples.
- b. For the first 20 determinations, results must fall within the acceptable range specified by the source of the standard.
- c. After 20 determinations, the mean value of the 20 determinations is used as the central line of a control chart.
- d. Warning limits are +/- two standard deviations.
- e. Control limits are +/- three standard deviations.

X. REFERENCES

- A. USATFMA Method 2C Cyclotrimethylenetrinitramine (RDX) in Soil and Sediment Samples, 12-3-80.
- B. USATFMA Method 8H Explosives in Water by HPLC, 12-27-82

XI. DATA

A. Off-the-Shelf Analytical Reference Materials
Characterization: Not Applicable

B. Initial Calibration

1. Response versus concentration data: See attached.
2. Response versus concentration graphs: See attached.
3. LOF Tests: Not applicable.
4. ZI Tests: Not applicable.

C. Daily Calibration

1. Response: Not applicable.
2. Required percentage or two standard deviation limits: Not applicable.

D. Standard Certification Samples

1. Tabulation and graph of found versus target concentrations: See attached.
2. LOF and ZI tests for the pooled data: See attached.
3. Calculated least squares linear regression line, confidence bounds, reporting limit, accuracy, standard deviation, percent imprecision, and percent inaccuracy: See attached.
4. Chromatograms: Attached